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Mini review

Protein secretion systems in *Pseudomonas aeruginosa*: A wealth of pathogenic weapons

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ABSTRACT

Pathogenic microorganisms have to face hostile environments while colonizing and infecting their hosts. Unfortunately, they can cope with it and have evolved a number of complex secretion systems, which direct virulence factors either at the bacterial cell surface into the environmental extracellular milieu or into the host cell cytosol. Six different classes of secretion systems have been described so far, currently identified as type I secretion system (T1SS) up to type VI secretion system (T6SS). The Gram-negative opportunistic human pathogen *Pseudomonas aeruginosa* possesses a broad panel of secretion systems. Five of the six secretion machines characterized in Gram-negative bacteria are at *P. aeruginosa* disposal, sometimes in several copies. All these machines are dedicated to the specific secretion of exoproteins, which display various activities useful for bacterial adaptation to the environment or for bacterial pathogenicity. This review will summarize the functional organization of these different secretion systems, which could constitute potential targets for therapeutic treatment of patients infected by one of the most potent nosocomial pathogens identified nowadays.

Introduction

In the course of an infection, bacterial pathogens are facing the hostile environment of the host. These microorganisms have to fight for their life by seeking out essential nutrients and avoiding eradication by the host immune system. In the game, they play with their host, they can also be skilful strategists and manipulate the host cell response for their own benefit. Most of these processes rely on a large number of proteins, which are deliberately released by the bacteria. These proteins could remain bound at the bacterial cell surface, be freed up into the extracellular medium or be injected into the host cell cytosol. We quote this protein release mechanism with the term secretion.

In Gram-negative bacteria, protein secretion is achieved after crossing the envelope formed by 2 hydrophobic barriers, the cytoplasmic, or inner membrane (IM), and the outer membrane (OM) delimiting the peptidoglycan-containing aqueous compartment, the periplasm (P). Molecular microbiologists have revealed over the last few decades the immense diversity of the mechanisms evolved by Gram-negative bacteria to secrete biologically active exoproteins. Six different classes of secretion systems have been described, which are identified as type I secretion system (T1SS) up to type VI secretion system (T6SS) (Economou et al., 2006). A type VII secretion system has been tentatively quoted in *Mycobacterium* species (Abdallah et al., 2009), but we wish to keep the "Type" nomenclature for Gram-negative bacteria and will thus not consider this system in our overview.

Secretion via T2SS and T5SS occurs in a two-step process including a stopover in the periplasm. Exoproteins using these pathways are synthesized as precursors, and their cleavable N-terminal signal peptide is used for targeting to the Sec or the Tat export machineries (Michel and Voulhoux, 2009). These macromolecular complexes, also called translocons, are involved in the passage of the exoproteins across the IM. The Sec system promotes export of unfolded proteins, whereas the Tat pathway promotes export of folded proteins, which need to accommodate cytoplasmic cofactors prior to their translocation into the periplasm. Further translocation across the OM is then achieved by the dedicated secretion systems, T2SS or T5SS. Secreted proteins using either one of these 2 systems are usually released in the extracellular medium or remain bound to the bacterial cell surface. In contrast, T1SS, T3SS, T4SS, and likely T6SS use a one-step mechanism, promoting the direct delivery of exoproteins into the extracellular medium (T1SS) or into the host cell (T3SS, T4SS, and T6SS). In all these cases, the proteins are not synthesized with a signal peptide but carry a so-called secretion

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signal, whose detailed characterization varies from one system to the other. In addition, alternative routes for secretion such as non-classical secretion (secretion without any identified secretion machinery) or outer membrane vesicles (OMVs)-mediated secretion have also been suggested (Bendtsen and Wooldridge, 2009), but these pathways have not been studied in molecular details, yet.

The opportunistic Gram-negative pathogen *Pseudomonas aeruginosa* is responsible for a wide range of human diseases, including septicaemia, pneumonia, and several other kinds of infection. This bacterium is implicated in acute and chronic infections, such as in the respiratory tract of cystic fibrosis (CF) patients. Except for the T4SS, all the other secretion machines described in Gram-negative bacteria have been identified in *P. aeruginosa* (Fig. 1). Thanks to this secretion arsenal, *P. aeruginosa* is able to produce and release a very broad variety of exoproteins. Most of these secreted proteins are toxins and hydrolytic enzymes, making *P. aeruginosa* an ideal model organism to study protein secretion and one of the most versatile pathogenic bacteria.

Type I secretion system or T1SS

Organization of the machinery

T1SS is one of the simplest bacterial secretion systems described to date. It requires an OM protein, an ABC (ATP-binding cassette) transporter, which is inserted in the IM and provides energy to the transport process, and an adaptor protein connecting these two components (Fig. 2) (Delepelaire, 2004; Holland et al., 2005). The OM component of the T1SS machinery is exemplified by the trimeric protein TolC. Structural studies on TolC have represented a real breakthrough in the field by revealing an unprecedented organization for an OM protein. Indeed, each subunit contains 4 β strands contributing to the formation of a single 12-stranded β barrel, which resembles other OM β barrels such as in porins. Moreover, several α helices projecting from this β barrel form a long tunnel of 100 Å into the periplasm (Koronakis et al., 2000). This observation sheds light on the concept of one-step translocation by explaining the absence of the requirement for a periplasmic intermediate.

T1SS substrates are targeted to the secretion machinery by a non-cleavable C-terminal secretion signal (Duong et al., 1996). These exoproteins are secreted in an unfolded form and interact, at least transiently, with both the ABC transporter and the adaptor components (Binet et al., 1997). Interaction of the secretion signal with the nucleotide binding domain (NBD) of the ABC transporter is thought to induce a conformational change promoting binding and hydrolysis of ATP at the NBD. The energy-driven conformational changes further move the T1SS substrate through the secretion system (Holland et al., 2005).

Type I secretion in P. aeruginosa

In *P. aeruginosa*, 2 T1SS have been studied experimentally. The Apr system consists of AprD (ABC transporter), AprE (adaptor), and AprF (OMF) and is involved in the extracellular secretion of the alkaline protease AprA and of AprX, a protein of unknown function (Table 1) (Guzzo et al., 1991; Duong et al., 2001). Like most secreted proteases, AprA is a recognized virulence factor involved in various *Pseudomonas* infections (Matsumoto, 2004). The other characterized T1SS is involved in utilization of iron and requires the *has* genes. The secreted protein HasAp (Table 1) is a haemophore, which binds haem from haemoglobin. The HasAp–haem complex is addressed to the OM receptor, HasR, which further drives haem internalization (Wandersman and Delepelaire, 2004). The T1SS used for HasAp secretion is classically consisting of HasD (ABC transporter), HasE (adaptor), and HasF (OMF) (Letoffe et al., 1998; Ma et al., 2003). The very low concentration of iron available to a pathogen within its host could thus be overcome by the secretion of haemophores, which capture haem from haemoglobin. In this context, HasAp is likely a crucial component for *P. aeruginosa* survival in the early stages of infection (Wandersman and Delepelaire, 2004).

Other T1SS have been described in *P. aeruginosa* (Ma et al., 2003), but in these cases no specific substrate has been reported so far.

Type II secretion system or T2SS

Organization of the machinery

The T2SS or secreton is one of the most versatile systems used by Gram-negative bacteria to secrete extracellular proteins in the surrounding environment (for review see Filloux, 2004; Michel and Voulhoux, 2009). It is unique in its ability to promote OM translocation of large multimeric exoproteins that are already folded in the periplasm. The T2SS is broadly conserved in Gram-negative bacteria and involves a set of 11–16 different proteins, which are given the generic names GspC-M, GspAB, GspN, GspO, and GspS, in which Gsp stands for "General Secretory Pathway". Based on homologies with the type IV pili assembly system (Peabody et al., 2003), the T2SS can be viewed as a molecular nanomachine consisting of 3 parts (Fig. 2). First, a protein platform is setting up the basis of the machine at the inner membrane (GspCEFLM components) and is using ATP as fuel for the GspE motor (ATPase). Second, a large channel constituted by a multimeric structure is embedded in the outer membrane (the secretin GspD). Third, a fimbrillar structure called the pseudopilus, which is formed by the assembly of the major pseudopilin GspG (Sauvonnet et al., 2000; Köhler et al., 2004) and capped with the minor pseudopilins GspI-K (Korotkov and Hol, 2008). The proposed role of the pseudopilus is to function as a piston, whose assembly is driven by the GspE ATPase, and which pushes exoproteins through the secretin channel out of the cell.

Type II secretion in P. aeruginosa

In *P. aeruginosa*, the Xcp (extracellular protein) T2SS is encoded by a set of 11 genes organized in two divergent operons, *xcpP* to *Q* and *xcpR* to *Z*, whereas a 12th gene, *xcpA/pilD*, is located outside of the 2 *xcp* operons (Fig. 1). *xcpA/pilD* encodes a peptidase involved in the maturation of the 5 T2SS pseudopilins (XcpT-X) and all other known pseudopilins and pilins of *P. aeruginosa* except for the Flp pilin, which is cleaved by FppA, the second prepilin peptidase of *P. aeruginosa* (Ruer et al., 2007). The ATPase XcpR directly interacts with XcpY and forms an IM complex with XcpZ and XcpS (Robert et al., 2005a). A fourth inner membrane protein, XcpP (Bleves et al., 1996), interacts at least transiently with the outer membrane secretin XcpQ. This interaction between the GspC (XcpP) and the GspD (XcpQ) components seems to be crucial and confers specificity to the secretion system (Gerard-Vincent et al., 2002). Interestingly, two other genes, *xphA* and *xqhA*, homologous to *xcpP* and *xcpQ*, respectively, have been identified (Michel et al., 2007). These orphan genes are located at a distant locus from the *xcp* gene cluster encoding components of the classical T2SS (Fig. 1). The *xphA* and *xqhA* gene products form a T2SS subunit that can associate with the XcpR-Z components of the classical T2SS to make a functional hybrid machinery. This machinery has its own specificity and even though it can promote secretion of most of the substrate of the classical Xcp T2SS, it is unable to translocate the aminopeptidase PaAP (Table 1) (Michel et al., 2007). More generally, it is proposed that the GspC component (XcpP or XphA) is a signal-transducing

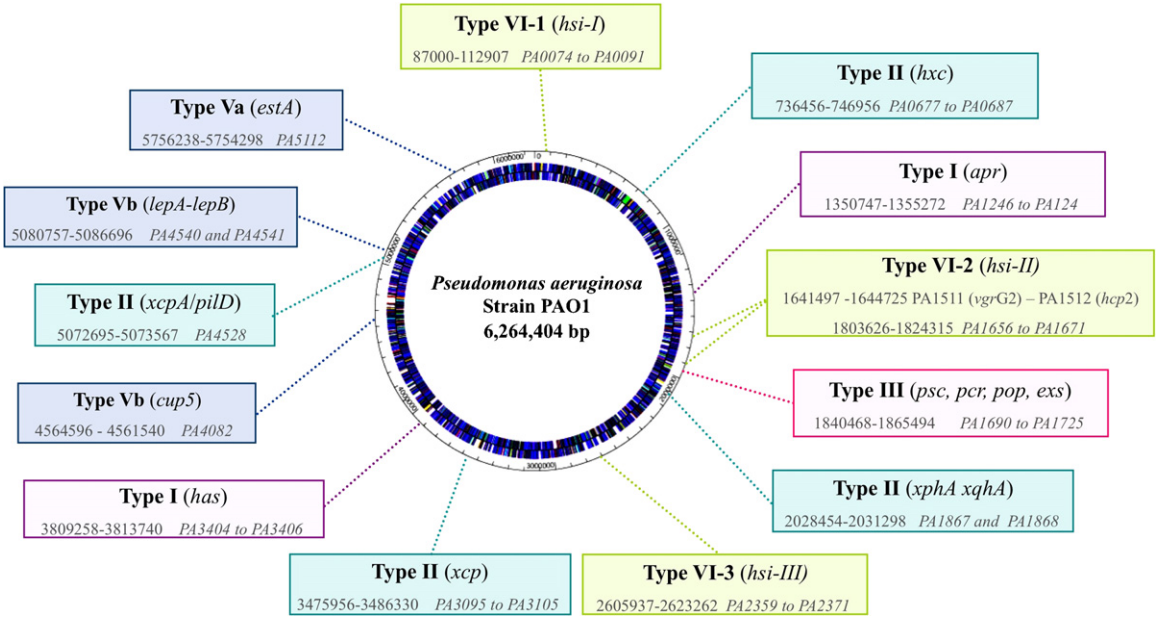

Fig. 1. Chromosomal localization of the various secretion machines found in *Pseudomonas aeruginosa* strain PAO1. Only experimentally studied secretion machines are represented accordingly with the colour code used in Fig. 2. Names of genes and/or locus tags (PA numbers) encoding the secretion machines as well as their genomic location are also represented according to the nomenclature used on the *Pseudomonas* website (http://v2.pseudomonas.com/).

protein switching on and off the secretion process (Robert et al., 2005b) by modulating the GspD (XcpQ or XqhA) central channel opening. The OM protein XcpQ is assembled as multimers of 12 subunits forming a ring-shaped structure with a central cavity of 90 Å diameter. The secretin thus constitutes a very large channel through which even folded exoproteins could pass.

Finally, five Xcp proteins (XcpT-X) are called pseudopilins. As for the type IV pilin PilA, pseudopilins possess a cleavable N-terminal sequence of 6 or 7 residues followed by a hydrophobic alpha-helix and a C-terminal soluble domain. Pseudopilins are first targeted and exported across the IM by the Sec/SRP pathway (Arts et al., 2007). The major pseudopilin XcpT is then assembled into a fimbrillar structure called pseudopilus (Durand et al., 2003; Alphonse et al., 2010). The precise mode of pseudopilus assembly as well as the involvement of the four minor pseudopilins (XcpU-X) in this process remains under debate. It is proposed that the XcpT-containing pseudopilus follows an assembly/disassembly cycle. Together with the four minor pseudopilins, it forms a piston, which pushes out proteins through the secretin channel towards the outside of the bacterial cell (Durand et al., 2005; Douzi et al., 2009). It should be noted that type IV pili retraction involves a second traffic ATPase, PilT. No such a component has been found to be required for the pseudopilus function, which suggests that it involves a different retraction process.

In addition to the initially characterized T2SS Xcp, *P. aeruginosa* possesses a second T2SS only functional in phosphate-limiting

Table 1
Exoproteins secreted by *Pseudomonas aeruginosa* PAO1 strain.

Secretion system	Secreted protein	Characteristics	Refs.[a]
T1SS (Apr)	AprA (PA1249)	Alkaline protease	Guzzo et al. (1991)
	AprX (PA1245)	Unknown	Duong et al. (2001)
	HasAp (PA3407)	Heme acquisition protein	Letoffe et al. (1998)
T2SS (Xcp)	LasB (PA3 724)	Metalloproteinase (Elastase)	Braun et al. (1998)
	Las A (PA 1871)	Staphylolytic and elastolytic	Braun et al. (1998)
	PlcH (PA0844)	Hemolytic phospholipase C	Voulhoux et al. (2001)
	PlcN (PA3319)	Non hemolytic phospholipase C	Voulhoux et al. (2001)
	PlcB (PA0026)	Phospholipase C specific of phosphatidyl-ethanolamine	Barker et al. (2004)
	CbpD (PA0852)	Chitin-binding protein	Folders et al. (2000)
	ToxA (PA1948)	AB Toxin, ADP-ribosyl transferase	Lu et al. (1993)
	PmpA (PA0572)	Putative metalloprotease	Voulhoux and Michel (unpublished)
	PrpL (PA4175)	Lysine specific endopeptidase (Protease IV)	Fox et al. (2008)
	LipA (PA2862)	Triacyl glycerol acyl hydrolase	Jaeger et al. (1994)
	LipC (PA4813)	Lipase	Martinez et al. (1999)
	PhoA (PA3296)	Alkaline phosphatase	Filloux et al. (1988)
	PaAP (PA2939)	Aminopeptidase	Braun et al. (1998)
T2SS (Hxc)	LapA (PA0688)	Alkaline phosphatase	Ball et al. (2002)
T3SS	ExoS (PA3841)	ADP-ribosyl transferase and Rho GTPase-activating protein (GAP)	Yahr et al. (1997)
	ExoT (PA0044)	ADP-ribosyl transferase and Rho GTPase-activating protein (GAP)	Yahr et al. (1997)
	ExoY (PA2191)	Adenylate cyclase	Yahr et al. (1998)
T5aSS	EstA (PA5112)	Esterase	Wilhelm et al. (1999)
T5bSS	LepA (PA4540)	Exoprotease	Kida et al. (2008)
	CupB5 (PA4082)	Hemagglutinin-like	Ruer et al. (2008)
T6SS (HSI-I)	Hcp1 (PA0085)	Nanotubes formation	Mougous et al. (2006)

[a] As referred, only secreted proteins experimentally demonstrated to use a defined secretory system are presented.

growth conditions, the Hxc (homologous to *xcp*) system (Ball et al., 2002). Although *hxc* gene organization is completely different from that of the Xcp system, the structural organization of the Xcp and Hxc machineries is thought to be very similar. In contrast to Xcp, the Hxc system has been shown to secrete only one substrate, the low-molecular-weight alkaline phosphatase LapA, produced under phosphate-limiting conditions.

Type II-dependent exoproteins and their role in virulence

P. aeruginosa can cause infections in patients compromised by underlying respiratory disease like bronchiectasis, CF, and diffuse panbrochiolitis (Kon et al., 1999). These infections are highly associated with the production of T2SS-dependent exoproteins listed in Table 1. These exoproteins are characterized by a broad diversity of activities and include proteolytic enzymes such as the elastase LasB (the major extracellular protease) (Olson and Ohman, 1992), the staphylolysin LasA (Olson and Ohman, 1992), the aminopeptidase PaAP (Cahan et al., 2001), PrpL or protease IV (Engel et al., 1998) as well as the predicted metalloprotease PA0572 (Voulhoux and Michel, unpublished). T2SS exoproteins do not present any conserved linear motif. They are folded in the periplasm, suggesting that the yet unknown secretion motif recognized by the secreton is likely to be conformational (Voulhoux et al., 2000).

Elastase encoded by the *lasB* gene degrades elastin, a major component of the lung tissue and critical for its elasticity. It also cleaves surfactant protein D (SP-D), which is involved in several immune functions such as bacterial aggregation, alteration of alveolar macrophage function, and regulation of bacterial clearance (Alcorn and Wright, 2004). More generally, although extracellular proteases are known to participate in *P. aeruginosa* keratitis (Matsumoto, 2004), their activity significantly contributes to tissue damage during respiratory infections (reviewed by Kipnis et al., 2006; De Bentzmann et al., 2000). Another family of T2SS-secreted proteins are the enzymes catalyzing lipid metabolism such as LipA and LipC lipases (Stuer et al., 1986; Martinez et al., 1999), phospholipases C, PlcH, and PlcN (Ostroff et al., 1990), which are targeted to eukaryotic membrane, and PlcB (Barker et al., 2004), a phospholipase specific for phosphatidyl-ethanolamine (Table 1). Two alkaline phosphatases, PhoA and LapA, are secreted by the Xcp and the Hxc T2SSs, respectively. The Xcp system also secretes a chitin-binding protein called CbpD that could have a role in pathogenicity as an adhesin mediating colonization of eukaryotic cells (Folders et al., 2000).

Exotoxin A (ToxA) is the only AB toxin with an intracellular target secreted by a T2SS in *P. aeruginosa*. Since it is a T2SS-dependent protein, ToxA is released into the extracellular milieu, but this enzyme is then capable of self-targeting to the eukaryotic target cell. The 3-dimensional structure of ToxA displays 3 distinct domains (Allured et al., 1986) characteristic of AB toxins. Domain I is responsible for cell recognition, domain II is involved in translocation of the toxin across the membrane of intracellular compartments, and domain III forms the catalytic domain leading to ADP-ribosylation of elongation factor 2, which results in protein synthesis inhibition and cell death.

Regulation of P. aeruginosa T2SSs

It has been shown that genes encoding the classical *xcp* machinery are under the control of a global regulatory system known as quorum sensing (QS) (Chapon-Herve et al., 1997). QS systems are complex cell-to-cell signalling systems that enable bacteria to sense cell density and to coordinate gene expression simultaneously within the whole bacterial population. In *P. aeruginosa*, the QS controls many of the virulence factors and involves at least 2 pairs of genes (*lasR/I* and *rhlR/I*) that both directly control expression of *xcp* and most exoprotein-encoding genes (Lazdunski et al., 2004). Interestingly, not all the genes encoding Xcp-dependent exoproteins are regulated by QS. For example, alkaline phosphatase, phospholipases C, PlcH, and PlcN are regulated by a 2-component regulatory system, PhoB/R, dependent on phosphate-limiting growth condition (Filloux et al., 1988). PhoB/R also controls the Hxc T2SS and its cognate exoprotein, LapA (Ball et al., 2002; Viarre and Voulhoux, unpublished). Recently, it has been reported that *hxc* genes could also be regulated by a cell surface signalling system designated as PUMA3 (Llamas et al., 2009).

Type III secretion system or T3SS

Organization of the machinery

A wide range of pathogens and symbionts are equipped with a T3SS that allows the injection of toxic proteins, called effectors, directly into the cytosol of host cells (Fig. 2). The contact between bacteria and eukaryotic cells leads to the translocation of the type III effectors both across the bacterial envelope and the eukaryotic plasma membrane in a one-step mechanism. The activity of these effectors, which are often eukaryotic-like proteins, may modify the host response by mimicking the activity of the endogenous proteins, which results in host subversion.

The T3SS apparatus, also called the injectisome, is related to the flagellum assembly machine, both in terms of similarities between the components but also in terms of overall architecture. Indeed, injectisomes of *Yersinia enterocolitica*, *Salmonella enterica*, or *Shigella flexneri* have been purified and visualized by electron microscopy as small syringes called also the needle complex, composed of an extracellular needle and a transmembrane cylindrical body (for review, see Cornelis, 2006).

Type III secretion in P. aeruginosa

In the *P. aeruginosa* PAO1 strain, five distinct operons located within a single genetic locus are involved in the biogenesis and the control of the type III secretion–translocation machinery (Fig. 1). In contrast, the genes encoding the effectors and their cognate chaperones are scattered around the chromosome. The *P. aeruginosa* needle-like filament is a straight hollow tube 60–80 nm long and 7 nm wide, made of PscF subunits (*Pseudomonas* secretion component) (Pastor et al., 2005; Soscia et al., 2007). The cytoplasmic PscE and PscG chaperones prevent PscF from premature polymerization in the cytoplasm (Quinaud et al., 2005). Furthermore, the PscP protein could serve as a molecular ruler controlling the PscF-needle length (Journet et al., 2003). Much less is known about the Psc proteins constituting the base of the needle complex, although some functions can be inferred from the closely related *Y. enterocolitica* T3SS. PscN is likely to be the putative energizer since it displays similarity with the bacterial F_0F_1 ATPase (Woestyn et al., 1994). PscJ is thought to be the major component of the inner base of the needle complex (Cornelis, 2006). PscC is a secretin that polymerizes, with the help of the PscW pilotin, to form a channel through the outer membrane (Koster et al., 1997), allowing the passage of the needle-like structure. One could note that secretins are found in 2 different secretion pathways, the T2SS and the T3SS.

Once the needle is assembled, the various exoproteins are transported through this conduit. Obviously, this is performed in a kinetic and ordered manner. The translocators PopB, PopD, and PcrV may be the first proteins transported to form a pore within the eukaryotic cell membrane, in the continuity of the PscF needle. PopB and PopD have hydrophobic domains, which allow their insertion into the eukaryotic cell membranes. PopB and PopD have been shown to polymerize in vitro and to form oligomeric rings, which

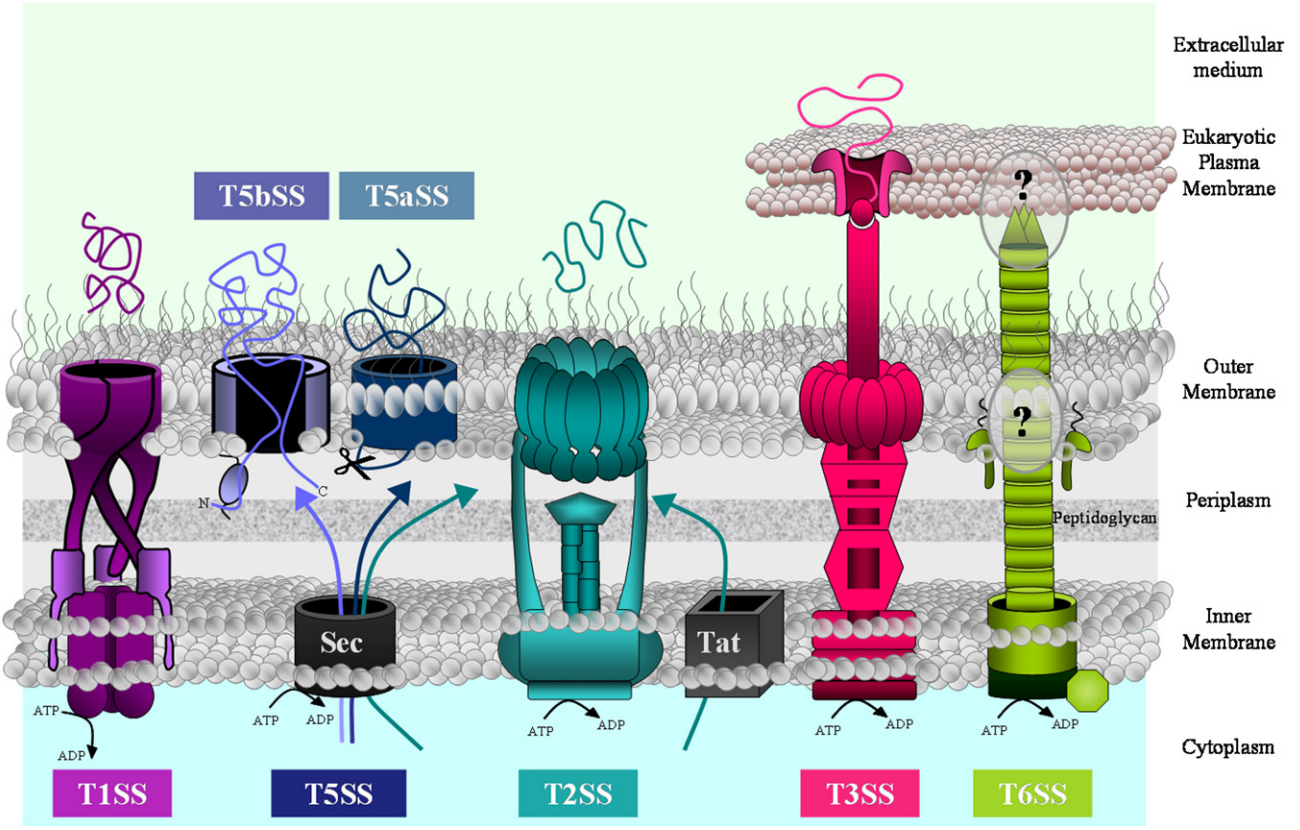

Fig. 2. Schematic representation of the different secretion systems found in *Pseudomonas aeruginosa* strain PAO1. Five of the six secretion pathways found in Gram-negative bacteria are present in *P. aeruginosa*. Protein transport across the bacterial envelope can be subdivided into Sec-independent and Sec/Tat-dependent pathways. Type II (T2SS)- and type V (T5SS)-secreted exoproteins are firstly exported to the periplasm by the Sec or the Tat system before crossing the outer membrane by their dedicated secretion pathway. In contrast, type I, type III, and type VI (T1SS, T3SS, T6SS) exoproteins are directly taken over in the cytoplasm by their cognate secretion machinery.

confirm that they may form pores (Schoehn et al., 2003). The size of these pores inserted in erythrocyte membranes after infection with *P. aeruginosa* was estimated 2.8–3.0 nm (Dacheux et al., 2001). Both translocators share a cytosolic chaperone, PcrH, that prevents premature interaction with the membranes. PcrV is necessary for the functional assembly of the PopB/D translocon, but does not seem to be a structural component of the pore, as it does not interact with the pore-forming proteins (Goure et al., 2004). PcrV is localized at the tip of the needle, where this scaffold protein may facilitate the assembly of the translocation pore (Mueller et al., 2005). Interestingly, specific antibodies directed against PcrV mediated protection against *P. aeruginosa* infections by presumably inhibiting effector injection (Sawa et al., 1999).

The four P. aeruginosa T3SS effectors and their chaperones

Once the translocon is inserted into the eukaryotic cell membrane, the T3SS effectors could then be transported through the needle and injected into the host cell by going through the PopB/PopD pore. The T3SS effectors are targeted to the secretion machinery in an unfolded-state via a non-cleavable N-terminal secretion signal (Cornelis, 2006). In vivo, T3SS functioning is triggered by the contact with the eukaryotic cell, which leads to effector injection. In vitro, T3SS activity could be triggered by Ca^{2+} ions chelation in the culture medium leading to effector release in the surrounding medium (Hornef et al., 2000). Strains of *P. aeruginosa* have different combinations of genes encoding T3SS effectors and can be divided into 2 groups. The so-called cytotoxic strains possess *exoU*, *exoT*, and sometimes *exoY* (e.g. strain PA103 or PA14), whereas invasive isolates possess *exoS*, *exoT*, and often *exoY* (e.g. strain PAO1 or PAK) (Wolfgang et al., 2003). The basis for the incompatibility in *exoU* and *exoS* occurrence within the same *P. aeruginosa* strain is not known, but the carriage of *exoS* is more prevalent among *P. aeruginosa* clinical isolates (Kulasekara et al., 2006). Moreover, the available set of T3SS effectors defines the potential phenotype of the strain during infection. ExoU-producing strains cause a rapid necrotic cell death, while ExoS-producing strains are rather internalized, which results in a delayed cell death resembling apoptosis (for reviews see Engel and Balachandran, 2009; Hauser, 2009).

ExoS and ExoT are bifunctional enzymes that include a GTPase-activating (GAP) function within the N-terminal domain and an ADP-ribosyltransferase activity within the C-terminal domain (Barbieri and Sun, 2004). They confer anti-phagocytotic capacities to *P. aeruginosa* mainly through their action on the actin cytoskelon. ExoY is an adenylate cyclase that leads to cyclic AMP (cAMP) accumulation in the host cell (Yahr et al., 1998). ExoU is a potent phospholipase A_2 harbouring a patatin-like active site responsible for acute cytotoxicity and lung tissue damages (for review see Sato and Frank, 2004). ExoU and ExoS prevent interleukin production by alveolar macrophages and modulate the early inflammatory response. Remarkably, all *P. aeruginosa* T3SS effectors require a eukaryotic cofactor for their activity, which highlights their specific function in the host.

In addition, ExoS and ExoT share the same cytoplasmic chaperone, SpcS (specific *Pseudomonas* chaperone) (Shen et al., 2008), and the ExoU chaperone is called SpcU (Finck-Barbancon et al., 1998). The essential function of T3SS chaperones remains a matter of debate. Among the various roles, one can mention (i) keeping the effector in an unfolded state competent for secretion, (ii) target-

ing to the T3SS apparatus, (iii) conferring kinetic and hierarchy to the secretion process, and (iv) preventing membrane localization during the secretion process (Cornelis, 2006; Letzelter et al., 2006).

Transcriptional regulation

The T3SS regulon consists of about 40 regulated genes, which are coregulated and under the control of ExsA, a member of the AraC family of transcriptional activators (Frank, 1997). ExsA binds to a defined nucleotide sequence upstream of the RNA polymerase binding site of *T3SS* gene promoters. Transcription of the *T3SS* genes is induced under calcium-limited growth conditions or following intimate contact of *P. aeruginosa* with host cells. This mechanism requires a cascade of interactions involving 4 proteins: (i) ExsA is the master regulator, (ii) ExsD is an anti-activator, which directly binds to ExsA and inhibits its activity (McCaw et al., 2002), (iii) ExsC is an anti-anti-activator, which binds to and inhibits ExsD (Dasgupta et al., 2004), but is also an ExsE chaperone, (iv) ExsE is a secreted anti-anti-anti-activator (Rietsch et al., 2005; Urbanowski et al., 2005). Under conditions that prevent secretion, ExsE remains intracellular, which favours formation of ExsE–ExsC and ExsA–ExsD complexes, thereby preventing ExsA-dependent transcription. Under low Ca^{2+} conditions or upon cell contact, ExsE is secreted or injected into host cells (Urbanowski et al., 2007). In the absence of ExsE, ExsC thus sequesters Exs D thereby making ExsA available to activate transcription of the *T3SS* genes.

Besides this dedicated pathway, the T3SS regulon is under the control of global regulatory systems involving cAMP biosynthesis (Wolfgang et al., 2003), 2-component systems RetS/LadS/Gac-Rsm (Goodman et al., 2004; Ventre et al., 2006; Mulcahy et al., 2006), and a variety of stresses (for a review see Yahr and Wolfgang, 2006). The T3SS is coregulated with the virulence factors required during the acute phase of infections, and its repression by QS suggests a requirement of T3SS functions before the establishment of a high cell density population thus at the early stage of an acute infection (Hogardt et al., 2004; Bleves et al., 2005). Conversely, active repression of the T3SS might contribute to the persistence of chronic infections.

Type V secretion system or T5SS

Organization of the machinery

T5SS is the simplest secretion pathway described so far, and it is widely represented in Gram-negative bacteria. It allows secretion through the bacterial envelope of large proteins associated with microbial adhesion and virulence. As for T2SS, T5SS is a two-step process. First, proteins cross the IM via the Sec export machinery; second, they are transported through an outer membrane (OM) channel formed by a β barrel protein/module. Finally, exoproteins either remain associated with the outer face of the OM or are released into the extracellular medium after a proteolytic cleavage. Two subtypes of T5SS exist in Gram-negative bacteria: the autotransporters (AT or T5aSS and T5cSS) and the 2-partner secretion (TPS or T5bSS) (Fig. 2).

Autotransporters (AT or T5aSS and T5cSS)

ATs are capable of "autosecretion" (Dautin and Bernstein, 2007; Henderson et al., 2004; Linke et al., 2006). Those proteins are modular proteins consisting of a signal peptide at the extreme N terminus, a β domain of 12 β strands at the C terminus, and in between, a "passenger" domain that harbours the catalytic domain of the protein. The signal peptide enables the export through the IM, and is then cleaved. Once in the periplasm, the C terminus domain is inserted into the OM and forms a β barrel leading either to the exposure of the passenger domain at the surface of the bacteria or to its release into the extracellular medium after cleavage by autoproteolysis or by a dedicated protease (Coutte et al., 2003; Hendrixson et al., 1997; Shere et al., 1997; van Ulsen et al., 2003). While β barrels of T5aSS are formed by one single monomer, β barrels of T5cSS are formed by a homo-trimer, in which each monomer contributes to 4 strands of the final β barrel (Yeo et al., 2004; Surana et al., 2004; Nummelin et al., 2004).

EstA is the only characterized AT in *P. aeruginosa* (Wilhelm et al., 1999). Its passenger domain possesses an esterase activity, i.e. EstA can hydrolyze glycerol esters with short- or long-chain fatty acid. Its catalytic site corresponds to the consensus motif GDSL, in which the serine residue is one of the 3 amino acids of the catalytic triad (serine–aspartate–histidine). The EstA passenger domain is not cleaved and therefore remains anchored at the bacterial surface. It was demonstrated that EstA is involved in the production of rhamnolipids, and an *estA* defective mutant was shown to be deficient in twitching, swarming, and swimming motilities, and thus in biofilm formation (Wilhelm et al., 2007).

Two other ATs, PA0328 and PA3535, have been predicted from the genome of the *P. aeruginosa* PAO1 strain (Stover et al., 2000; Filloux et al., 2003) (Fig. 1). These proteins may encode a putative aminopeptidase and a serine protease, respectively (http://v2.pseudomonas.com/).

Two-partner secretion (T5bSS)

The TPS mechanism is very similar to the AT, except that the passenger domain and the β domain are independent proteins, named TpsA and TpsB, respectively (Hodak and Jacob-Dubuisson, 2007; Mazar and Cotter, 2007). TpsA contains a TPS secretion motif and a functional/catalytic domain, whereas in TpsB, the β domain is preceded by one or several POTRA domains (polypeptide transport-associated). POTRA domains are proposed to be involved in protein–protein interaction, and in the case of TpsB, in the recruitment of the TpsA protein. They are found among members of the Omp85/BamA protein superfamily to which the TpsBs belong. The Omp85/BamA members are more generally involved in the assembly and proper localization of OM proteins (Voulhoux and Tommassen, 2004).

TpsA and TpsB proteins cross the IM via the Sec export pathway. The TpsA, synthesized as a pre-pro-protein, is converted into a pro-protein after maturation of its signal peptide. The TpsB inserted into the OM recruits the TpsA via one of its POTRA domains that interacts with the TPS motif of the TpsA (Clantin et al., 2004; Hodak and Jacob-Dubuisson, 2007). Once in the OM, the pro-protein TpsA undergoes an additional cleavage, leading to the mature TpsA protein (Jacob-Dubuisson et al., 2001), which remains either anchored at the bacterial surface or is released into the extracellular milieu.

TpsB and AT β barrels display different, but typical features. In addition to the presence of the POTRA domain, TpsB β barrels are composed of 16 β strands (Clantin et al., 2007; Hodak and Jacob-Dubuisson, 2007), whereas ATs are 12-stranded β barrels (Oomen et al., 2004). Even if the primary sequence of all TpsB translocators is quite different, the structural conformation of those proteins is very well conserved (Yen et al., 2002).

Six TPS clusters have been annotated on the genome of the PAO1 strain of *P. aeruginosa*. Five are complete, *tps1*: *PA0040–PA0041*; *tps2*: *PA0690–PA0692*; *tps3*: *PA2462–PA2463*; *tps4*: *PA4540–PA4541*; *tps5*: *PA4624–PA4625*; and one of them is composed of an orphan *tpsA* gene: *tpsA6* (*PA4082*). Only two, TPS4 and TPS6, have been characterized (Fig. 1). The TpsB4 transporter (PA4540) named LepB is required for the secretion of the TpsA4 (PA4541) protein, LepA. LepA is a protease that activates the critical transcription factor NF-κB for host inflammatory and immune responses through digestion of human specific receptors (PAR-1, -2, or -4) (Kida et al., 2008).

It should be pointed out that LepA was detected in the extracellular medium of clinical isolates, but not of the laboratory strain PAO1.

The *tpsA6* gene, also called *cupB5*, is located within the *cupB* gene cluster encoding chaperone usher pathway components involved in the assembly of CupB fimbriae. The CupB3 usher makes a pore in the OM through which pilin subunits transit to form fimbriae at the cell surface. Interestingly, the CupB3 usher is also required for the secretion of the TpsA6/CupB5 protein that lacks a cognate TpsB transporter (Ruer et al., 2008). Since CupB3 is an usher protein carrying a POTRA domain, it has been called a P-usher (for POTRA-containing usher). In contrast to what is proposed for TpsB POTRAs, the POTRA of the P-usher (CupB3) is more specifically required for proper folding of the TpsA (CupB5). If the P-usher/CupB3 lacks its POTRA, TpsA/CupB5 is misfolded. The misfolded TpsA/CupB5 blocks the CupB3 channel and prevents further assembly of CupB fimbriae. Thus, the CupB3 POTRA domain is crucial to properly coordinate fimbriae assembly and TpsA6/CupB5 secretion (Ruer et al., 2008). The TpsA6/CupB5 protein is a hemagglutinin-like protein that may be interwoven with the CupB fimbriae (Ruer et al., 2008).

Type VI secretion system or T6SS

Organization of the machinery

The most recently discovered secretion pathway in *P. aeruginosa* is the type VI secretion system (T6SS), which is widely spread in Gram-negative bacteria. Substrates for this secretion system were first described in *Vibrio cholerae* by Pukatzki et al. (2006) who demonstrated that proteins lacking a signal peptide, called Hcp (hemolysin-coregulated protein) and VgrG (valine–glycine repeat), require a functional T6SS for their release into the extracellular medium.

However, several lines of evidence suggest that secreted Hcp- and VgrG-like proteins can also be considered as extracellular components, which are part of the T6SS machinery. On the one hand, the crystal structure of the *P. aeruginosa* Hcp1 protein reveals a hexameric-ring structure (Mougous et al., 2006), homologous to the tube domain of the T4 phage tail, the protein gp19 (Leiman et al., 2009). Moreover, Hcp1 rings readily polymerize in solution and form nanotubes with an internal diameter of 9 nm (Ballister et al., 2008; Leiman et al., 2009). On the other hand, the conserved N-terminal region of VgrGs shares similarities with the gp5 and gp27 T4 phage proteins. gp5 and gp27 multimers constitute the bacteriophage tail spike used for puncturing the bacterial envelope and to inject DNA into the cytoplasm (Kanamaru et al., 2002). The available crystal structure of the *Escherichia coli* CFT073 VgrG confirms the structural similarity between VgrGs and the gp5–gp27 complex (Leiman et al., 2009). It has been proposed that this VgrG domain sits at the tip of the Hcp nanotubes and may contribute to the perforation of the bacterial cell envelope and/or the host cell membrane (Filloux et al., 2008; Pukatzki et al., 2007, 2009). Interestingly, besides this peculiar function, VgrGs may also act as genuine effectors thanks to an additional C-terminal extension found in only some VgrGs, so-called "evolved VgrGs", (Pukatzki et al., 2007, 2009; Filloux et al., 2008). For example, the C terminus of the VgrG-1 protein of *V. cholerae* resembles the actin cross-linking domain of the RtxA toxin (Pukatzki et al., 2007) and was shown to be delivered into macrophages after phagocytosis of the bacteria (Ma et al., 2009).

Other characterized T6SS components are: a ClpV ATPase belonging to the AAA$^+$ family, a regulatory FHA (forkhead-associated) domain protein that is the target of a couple of Ser/Thr kinase and phosphatase, DotU- and IcmF-like proteins homologous to type IV secretion system (T4SS) components, and an outer membrane lipoprotein (Filloux et al., 2008; Aschtgen et al., 2008). Strikingly, most of the other T6SS components are predicted to have a cytoplasmic localization.

T6SS in P. aeruginosa

The genome of the *P. aeruginosa* PAO1 strain contains 3 loci encoding T6SS components, called *HSI-I*, *HSI-II* and *HSI-III*, with about 15–20 genes each (Fig. 1). The post-translational control of the *P. aeruginosa* HSI-I machinery by threonine phosphorylation has been well documented (Mougous et al., 2007; Hsu et al., 2009). Upon phosphorylation of the Fha1 protein by the Ser/Thr kinase PpkA, the cytoplasmic complex formed by Fha1 and the ATPase ClpV1 is directed to the membrane site of the HSI-I apparatus (Hsu et al., 2009). This activation may be coupled to the ClpV1-dependent disassembly of tubule-like structures, constituted by two T6SS components of unknown function, as shown in *V. cholerae* (Bonemann et al., 2009; Filloux, 2009).

The secretion of Hcp1 is so far the only example of a T6SS substrate in *P. aeruginosa*, and the Hcp1 protein can be detected in the sputum of cystic fibrosis patients (Mougous et al., 2006). The secretion of this putative structural component cannot account for the role of the HSI-I machinery in *P. aeruginosa* virulence and persistence within the host (Potvin et al., 2003), and VgrGs or other HSI-I-dependent exoproteins are currently under study. Moreover, animal and plant infection studies reveal that both HSI-II and -III play important roles in *P. aeruginosa* virulence (Lesic et al., 2009). Furthermore, the phenotype of a double mutant affected in the two systems suggests that these machineries may functionally compensate for each other in the pathogenesis process.

Transcriptional regulation

The three *P. aeruginosa* T6SS loci appear to have distinct evolutionary origins (Bingle et al., 2008). In agreement with this, they are differently regulated, which suggests that they act in different context and may perform different roles. The *HSI-I* gene cluster is regulated by 2-component systems and sRNAs (Goodman et al., 2004; Mougous et al., 2006), while *HSI-II* and *-III* are not. Moreover, QS represses *HSI-I*, while it activates *HSI-II* and *-III* as many other virulence such T1SS and T2SS (Lesic et al., 2009).

Conclusion

P. aeruginosa secretion systems are diverse and complex. Such systems can be as simple as the T5aSS/T5cSS (one single accessory protein supporting the secretion of one exoprotein), or the T1SS (3 different accessory proteins for 1–2 substrates). They can also involve much more complex machineries such as the T2SS (11 proteins assembled in a supramolecular structure with 3 functional units dedicated to the secretion of at least 13 exoproteins), or the T3SS (coordinated production of about 40 accessory proteins needed for the injection of 3 effectors into target cells). The T6SS is also of high complexity, with 12–25 accessory proteins constituting the machinery. In contrast to other systems, the identity of the T6SS-dependent secreted proteins is not yet obvious, since Hcp1 is mostly considered as a component of the machinery rather than a real T6SS substrate. Hcp1 forms nanotubes, and in this respect it may be compared to the T3SS needle or the T2SS pseudopilus.

The various degrees of complexity observed between the secretion machines are likely a matter of evolutionary origin rather than a deliberate choice of making things complex or not. Interestingly, some of these complex apparatuses are similar to machines involved in assembly of cell surface appendages like type IV pili (T2SS) or flagella (T3SS). It is thus tempting to speculate that at some points, these open gates to the external medium have been

hijacked and used to release proteins extracellularly. The most simple ones, like the T5SS (AT or TPS), might have taken advantage of the β-barrel structure of porins or other OM proteins, to accommodate the release of larger substrates.

The different secretion machineries are dedicated to specific exoproteins, with all kind of activities useful for bacterial adaptation to the environment or to the host. All these systems differ by their mode of action. This diversity allows the secretion of either folded (T2SS) or unfolded proteins (T1SS, T3SS). The diversity also fine-tunes the final localization of the exoproteins, which is either at the bacterial cell surface, into the extracellular milieu, or into target host cells. Among the 5 secretion systems present in *P. aeruginosa*, at least 2, the T3SS and the T6SS, are believed to require a direct contact between the bacteria and the eukaryotic target cells in order to be effective.

We have shown along this review the great wealth of secretion machines and secreted proteins found in the model bacterium *P. aeruginosa*. This organism is originally an environmental strain that can be found in a broad diversity of niches such as on plants, in soil or humid environment for example. Such versatility may explain why *P. aeruginosa* has so many systems allowing growth and survival in so much distinct environments. Not only *P. aeruginosa* has evolved hydrolytic and scavenging systems to acquire nutriments and compete with other microorganisms, but it may have needed defence mechanisms against other organisms such as amoeba or nematodes, which are also found in those environments. The complexity and the variety of the regulatory mechanisms involved in controlling and coordinating the production of all these secretion systems in a timely manner is also a reflection of this environmental diversity.

Once *P. aeruginosa* entered in contact with immunocompromised human hosts, especially at the hospital, it is easy to imagine that, because of the availability of such an impressive secretion arsenal, it became one of the most dangerous opportunistic pathogens and is highly ranked in the classification of nosocomial agents. However, the potent guns that constitute secretion machineries may also represent its Achille's heel. Indeed, several research lines use these molecular weapons as targets for the development of antibacterials, in order to block their functioning and avoid dissemination of virulence factors.

Note added in proof

The authors would like to mention the three following publications about novel secreted proteins of *P. aeruginosa* released during the publication process of this review: (i) Hood et al. (2010) identified five substrates of the H1-T6SS machinery, the proteins Tse1 to Tse3 (type six exported 1-3), and the VgrG1 and VgrG3 proteins. Tse2 was found to be the toxin component of a toxin-immunity system that arrests the growth of prokaryotic and eukaryotic cells. (ii) Borlee et al. (2010) characterized the CdrA protein (cyclic diguanylate-regulated TPS partner A), a secreted adhesin of the tps5 cluster, as a key structural component of the *P. aeruginosa* biofilm extracellular matrix. (iii) Salacha et al. (2010) described PlpD (patatin-like protein D), a lipolytic enzyme, as the archetype of a novel type V secretion system called T5dSS, discovered in *P. aeruginosa* and scattered among pathogenic and environmental bacteria.

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